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In vivo measurement of the size of oil bodies in plant seeds using a simple and robust pulsed field gradient NMR method

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Abstract An easy to implement and convenient method to measure the mean size of oil bodies (OBs) in plant seeds is proposed using a pulsed field gradient nuclear magnetic resonance (PFGNMR) approach. PFGNMR is a well-known technique used to study either free or restricted diffusion of molecules. As triacylglycerols (TAG) are confined in OBs, analysis of their diffusion properties is a well-suited experimental approach to determine OB sizes. In fact, at long diffusion time, TAG mean squared displacement is limited by the size of the domain where these molecules are confined. In order to access the OB size distribution, strong intensities of magnetic field gradients are generally required. In this work we demonstrate for the first time that a standard liquid-phase NMR probe equipped with a weak-intensity gradient coil can be used to determine the mean size of OBs. Average sizes were measured for several seeds, and OB diameters obtained by PFGNMR were fully consistent with previously published values obtained by microscopy techniques. Moreover, this approach provided evidence of TAG transfer through the network of interconnected OBs, which is dependent on the ability of adjacent membranes to open diffusion routes between OBs. The main advantage of the NMR method is that it does not require any sample preparation and experiments are performed with whole seeds directly introduced in a standard NMR tube.

Keywords PFGNMR · Mature seeds · Oil body size · In situ determination · Storage lipids · Lipid quantification · TAG

Abbreviations

CPMAS	Cross-polarization magic-angle spinning
DW	Dry weight
FW	Fresh weight
MSD	Mean squared displacement
NMR	Nuclear magnetic resonance
OB	Oil body
PFGNMR	Pulsed field gradient NMR
TAG	Triacylglycerols

Introduction

Lipids can be one of the most abundant constituents of seeds in oleaginous crops. They are mainly found as triacylglycerols (TAG). TAG are very important components in the biochemistry of plants (Chapman et al. 2012). Besides their relevance in human and animal feeds, they appear as a potential source of renewable and sustainable energy that does not participate in atmospheric CO_2 enrichment (Pinzi et al. 2009; Russo et al. 2012; Atabani et al. 2013). They

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can be used to reduce simultaneously global warming and depletion of fossil-fuel resources.

In seeds, TAG are stored in small subcellular spherical structures—oil bodies—composed of TAG surrounded by a layer of phospholipids and structural proteins which ensure oil body stability. The average OB diameters of the majority of vegetable seeds among common crops range from 0.5 to 2 microns (Huang 1992, 1996; Murphy 1993; Tzen et al. 1993; Murphy et al. 2001; Hsieh and Huang 2004). The range of their size distribution is variable and depends on taxonomy and abiotic factors. For many applications, in either fundamental or applied research, assessing these morphological parameters with a robust and fast technique could provide a breakthrough in this field and may be of primary importance for seed and oil producers.

Most data on oil body size have been obtained by electron or optical microscopy. Although the results are reliable, the sample preparation is very time consuming and not straightforward. The steps of sample fixation, sectioning, and staining are required for most microscopy techniques. Light scattering and microscopy techniques are also used for measurement of the size and behavior of isolated OBs (Jolivet et al. 2013). The pertinent third-harmonic generation microscopy technique allows study of OBs in nontreated seeds, but it has not been developed as a routine method up to now (Debarre et al. 2006). Furthermore, for all quantitative microscopy measurements, a statistical approach is essential for the interpretation of the results (Miquel et al. 2014).

In the context of seed characterization, NMR spectroscopies have appeared as valuable alternative methods to investigate seed features at different length scales. At molecular level, high-resolution solid-state ^{13}C or ^{1}H NMR studies using magic-angle spinning of the sample have undoubtedly appeared as a rapid and noninvasive method to study the components of solid and mobile seed domains (Odonnell et al. 1981; Bardet et al. 2001, 2006; Terskikh et al. 2005). On a large spatial scale (30 μm × 30 μm), magnetic resonance imaging (MRI) has been used for quantitative imaging of oil storage without direct access to OB sizes (Neuberger et al. 2008; Fuchs et al. 2013). To investigate their morphological and dynamical properties, liquid-state NMR is an appropriate method; for instance, the amount of mobile TAG can be directly measured and the sizes of OBs revealed by using NMR spectroscopy with pulsed magnetic field gradients.

In fact, pulsed field gradient nuclear magnetic resonance (PFGNMR) has been demonstrated to be a powerful method to measure the self-diffusion coefficient and to characterize the restricted diffusion in mesoporous media and confined systems (Stejskal and Tanner 1965; Tanner and Stejskal 1968; Price 1997, 1998). PFGNMR and also permanent field gradient methods have been applied to TAG moieties to characterize the morphology of OBs in seeds (Fleischer et al. 1990; Zakhartchenko et al. 1998; Carlton et al. 2001; Guillermo and Bardet 2007). A complete methodological approach has been reported, showing that it was possible to use PFGNMR experiments to determine both the average size of oil bodies and their size dispersion (Guillermo and Bardet 2007). It is clear that TAG diffusion obeys a restricted process due to their confinement in small, micrometric volumes. Analysis of the PFGNMR signal from confined TAG allowed the size dispersion of the OBs to be determined. In this work, a strong gradient NMR probe dedicated to diffusion measurements was used (max. gradient ~10 T m^{-1}). To apply this method as a robust laboratory technique for biological applications, it is necessary to extend it to standard liquid-phase NMR probes equipped with weak gradients (max. gradient ~0.5 T m^{-1}). Such gradients are commonly used to select appropriate coherence pathways in multipulse experiments. They are also used in liquid-phase NMR to edit selectively the subspectrum of each component of a mixture according to the self-diffusion coefficients in the experimental technique known as diffusion-ordered spectroscopy (DOSY) (Morris and Johnson 1992). The aim of the present work is to demonstrate that such equipment is adequate to measure the mean diameters of oil bodies inside seeds. The main features of the method we propose are the following: (1) It does not require any dedicated sample preparation; (2) It is a fast method; (3) It can be implemented on NMR spectrometers available in numerous biology laboratories. One can notice that the experimental constraints specific to the method match the conditions of TAG contained in oil bodies. First, OB sizes coincide with the spatial resolution (a few microns) of the PFGNMR experiment. Second, the proton relaxation rates of TAG are long enough to allow their diffusion properties to be characterized at long diffusion times. Moreover, as the TAG amount in seeds can be also quantified using direct proton excitation (Mantese et al. 2006; Samii-Saket et al. 2011), its correlation with the size of oil bodies can also be achieved.

Materials and methods

Plant material

Seeds of sunflower (*Helianthus annuus*), sesame (*Sesamum indicum*), mustard (*Brassica nigra*), walnut (*Juglans regia* L.), and lettuce (*Lactuca sativa*), all in a dormant physiological state, were used for these experiments. Sesame, mustard, and lettuce seeds were put directly into a 5-mm-diameter NMR tube with no other preparation. The fresh weight (FW) of seed samples was between 30 and 50 mg. The height of seeds in the tube was about 6–9 mm. For

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sunflower, the pericarps were removed and the seeds analyzed one by one. After the NMR experiment, the germination ability of the seeds was verified to be unaffected (germination on water-imbibed Whatman paper). For analyses of walnut (*Juglans regia* L.), a homogeneous piece of kernel compatible with a 5-mm NMR tube was prepared (~40 mg). The dry weight (DW) of seeds was determined after 48 h drying at 60 °C.

NMR experiments

All NMR measurements were performed at 298 K with a Bruker AVANCE 500 spectrometer equipped with a 5-mm BBI-xyz-gradient probe. This equipment cannot detect the solid domains of seeds, which requires the CPMAS technique and corresponding probe, so only NMR spectra of mobile (liquid-state) species were recorded. For TAG quantification, the sample volume was smaller than the volume of the radiofrequency coil of the probe. To take into account the different filling factors of the coil, probe settings and pulse calibration were checked for each sample. The receiver gain was kept constant for a series of seeds and the oil reference. The experiments were done with a recycling delay five times longer than the longest spin–lattice relaxation time. The NMR probe sensitivity was calibrated by using known amounts of olive oil placed in an NMR tube, which was used as a reference for the quantification of TAG in seeds. The ^{1}H NMR spectra were referenced by setting signal *#4* (Fig. 2b) assigned to α-protons of TAG glycerol to 4.08 ppm with respect to tetramethylsilane (TMS) set to 0 ppm (Sacchi et al. 1997). The calibration was carried out by adding to a sunflower oil sample a small volume (5 % v/v) of $CDCl_3$ containing 1 % (v/v) TMS.

In pulsed field gradient ^{1}H NMR mode, the diffusion filtered spectra were recorded with the standard bipolar pulse sequence (Fig. 1) combining a radiofrequency stimulated echo of the magnetization and a bipolar pulsed gradient sequence (Cotts et al. 1989; Fordham et al. 1994; Wu et al. 1995). The amplitude g of the trapezoidal gradient pulses was varied from 5 to 98 % of the maximum amplitude (0.48 T m^{-1}). This trapezoidal shape of the gradient pulse, of which the integral is 90 % of a rectangular δ pulse, was taken into account to calibrate the gradient value. The diffusion coefficients D were calculated according to the equation

$$I/I_0 = \exp(-kD), \tag{1}$$

with $k = (\gamma_H g\delta)^2(\Delta - \delta/3 - \tau/4)$, where I is the spectrum integral at g and I_0 is the integral value extrapolated to zero gradient, γ_H is the hydrogen gyromagnetic ratio (2.6752×10^8 rad s^{-1} T^{-1}), D (m^2 s^{-1}) is the diffusion coefficient of the considered species, and δ and Δ are the

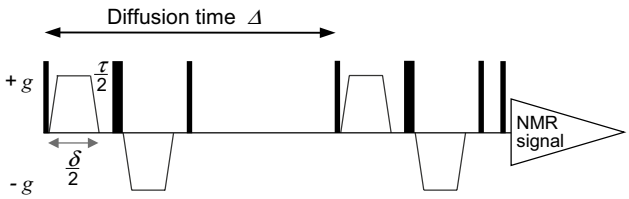

Fig. 1 ^{1}H PFGNMR sequence used in this work (Wu et al. 1995). Two elementary pulse blocks [$\pi/2$, $\delta/2$, $\tau/2$, π, $\delta/2$, $\tau/2$, $\pi/2$] are separated by a long diffusion time ($\Delta - \tau - \delta$). The rise time and fall time of gradient pulses are equal to 10 % of the total pulse duration. The sequence corresponds also to the so-called type 13 interval (case 1) reported in Cotts et al. (1989) for minimizing the effects of residual steady gradients

gradient pulse duration and delay during which the diffusion is observed. In this sequence, delays ($\delta + \tau$) and [$\Delta - (\delta + \tau)$] are the evolution times for the transverse, T_2, and longitudinal, T_1, relaxations, respectively. The diffusion time range available for a given molecular system depends on its longitudinal relaxation rate properties, I_0 being proportional to $\exp[-2(\delta + \tau)/T_2 - (\Delta - \delta - \tau)/T_1]$. The T_1 and T_2 values measured at 500 MHz with the integral of TAG spectra ($T_1 \approx 0.5$–0.6 s and $T_2 \approx 0.03$ s at 298 K) were actually a favorable experimental feature: long T_1 is necessary for PFGNMR measurement at long diffusion time Δ, and T_2 is long enough to avoid strong attenuation of the signal during the delay ($\delta + \tau$). Moreover, the monoexponential behaviors of both T_2 and T_1 show that a single TAG moiety is monitored by the PFGNMR experiment. The experiments were done with $\delta/2$ ranging between 1.5 and 3 ms, $\tau/2$ equal to 0.2 ms, and Δ varying from 10 ms to 1 s. Data were analyzed using commercial software (Bruker Biospin).

Results and discussion

General features of ^{1}H NMR spectra

The typical ^{1}H NMR spectra obtained respectively for a sunflower seed and pure sunflower oil are compared in Fig. 2. Despite the observed line broadening, the ^{1}H NMR spectrum of the sunflower seed (Fig. 2a) superimposes on the spectrum of the mobile TAG molecules in bulk oil (Fig. 2b).

The NMR signal broadening of lipids in the sunflower seeds is mainly due to a distribution of the magnetic field intensity inside the seed. This field intensity dispersion is a consequence of the heterogeneous consistency of the sample. The high spatial homogeneity of the magnetic field typically required for homogeneous liquid samples cannot be achieved here, and spectral resolution is lost. For such

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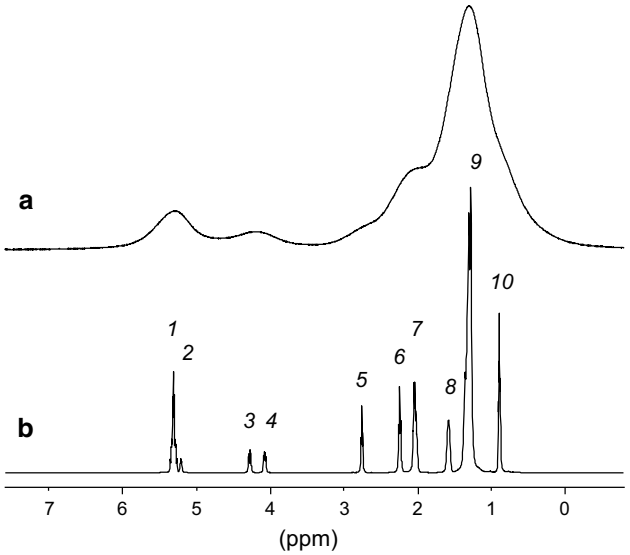

Fig. 2 500-MHz ^{1}H NMR spectra of TAG molecules in a sunflower seed (**a**) and in sunflower oil (**b**) with the following NMR assignment: (*1*) –C**H**=C**H**–, (*2*) –C**H**O–, (*3*, *4*) –C$\mathbf{H_2}$O– of glycerol moiety, (*5*) =CH–C$\mathbf{H_2}$–CH=, (*6*) –O–CO–C$\mathbf{H_2}$–, (*7*) -HC=CH–C$\mathbf{H_2}$–$(CH_2)_n$–, (*8*) –O–CO–CH_2–C$\mathbf{H_2}$–, (*9*) –$(\mathbf{CH_2})_n$–, (*10*) –C$\mathbf{H_3}$

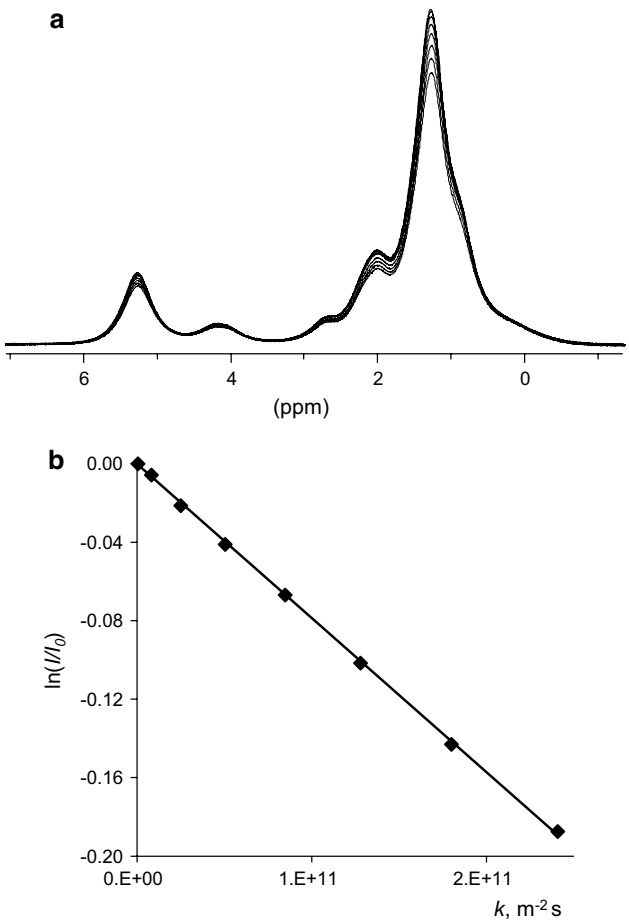

Fig. 3 **a** 500-MHz ^{1}H NMR stack-plot of sunflower seed spectra recorded by PFGNMR with increasing gradient strength. Experimental conditions in Fig. 1 and for Eq. 1 are: $\Delta = 400$ ms, $\delta/2 = 3$ ms, $\tau/2 = 0.2$ ms, g varying from 5 to 98 % of the maximum amplitude (0.48 T m^{-1}). The diffusion coefficient was calculated by fitting experimental data with Eq. 1, $D_{meas} = (7.9 \pm 0.2) \times 10^{-13}$ m^2 s^{-1}. **b** Semilogarithmic plot of the attenuation function I/I_0 highlighting the k-range used to calculate D_{meas}

samples, magic-angle spinning would be the classical way to recover high-resolution features (Bardet et al. 2001).

Proton pulsed field gradient NMR: OB size determination

As mentioned previously, the vegetable seed medium is not homogeneous enough for high-resolution NMR requirements. This means that, for Eq. 1 to remain valid, the PFGNMR sequence has to minimize the effects of static field gradients coming from the heterogeneous structure of the sample. The PFGNMR sequence we used was one of the sequences known to be efficient for removing the contribution of static gradients (Cotts et al. 1989). In the PFGNMR experiments reported in the present work, the attenuation of spectra integrals (Eq. 1) was obtained by increasing gradient intensities while keeping constant the delays of the PFGNMR sequence (Fig. 1). A typical series of spectra recorded in such conditions is shown in Fig. 3a.

The relatively small attenuation of the NMR signal is a consequence of the weak gradients available with the chosen NMR equipment. However, let us recall that we focus here on the determination of the mean size of oil bodies only. We do not aim to access the size distribution, which would necessitate stronger signal attenuation as obtained by using specific equipment such as a high-gradient NMR probe dedicated to diffusion measurements (Guillermo and Bardet 2007). It is worth noting that the beginning of the attenuation curve, used in this work for OB size determination, is fully relevant to characterize TAG diffusion properties by a diffusion coefficient D_{meas} provided that the attenuation obeys a pure exponential shape versus the factor k. Using Eq. 1, with a diffusion delay set to 400 ms, the TAG diffusion coefficient was found to be $D_{meas} = (7.9 \pm 0.2) \times 10^{-13}$ m^2 s^{-1} (Fig. 3b). With the same experimental conditions, the self-diffusion coefficient of sunflower oil was equal to $(1.13 \pm 0.03) \times 10^{-11}$ m^2 s^{-1}.

Assuming that all TAG molecules are embedded in oil bodies, the low diffusion coefficient measured for TAG inside seeds compared with its value in bulk oil points to an apparent slowing down of the molecules' dynamics that can be attributed to restricted molecular motions. In a Brownian diffusion process, the product $D\Delta$, where D is the self-diffusion coefficient, is proportional to the mean squared displacement (MSD) of molecules ($\langle r^2 \rangle \sim D\Delta$)

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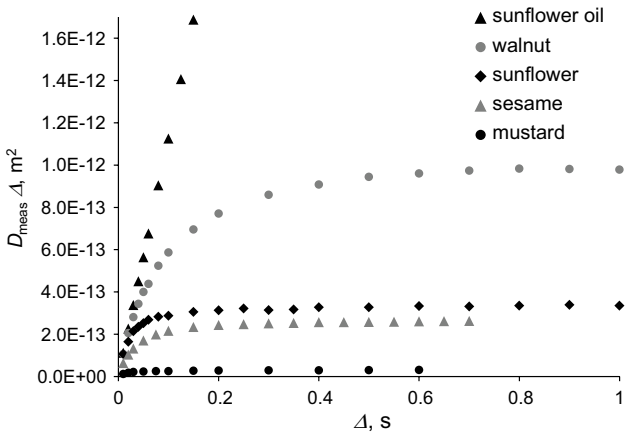

Fig. 4 Self-diffusion of TAG in bulk sunflower oil and restricted diffusion of TAG in oil bodies in four seeds. The mean squared displacement $D_{meas}\Delta$ is plotted versus the diffusion time Δ. The corresponding OB mean diameters are given in Table 1

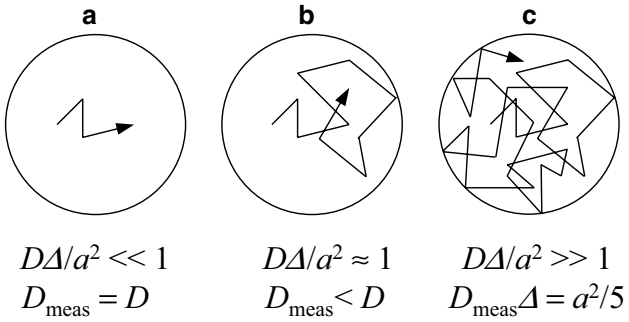

Fig. 5 Schematic representation of TAG molecule diffusion in a spherical OB of radius a

(Price 1997). Thus, to evidence an effect of confinement, the experiment described in Fig. 3 was repeated, changing the diffusion time Δ, in order to find an upper limit of the MSD value. In Fig. 4 the product $D_{meas}\Delta$ is plotted versus the diffusion time for TAG in OBs and bulk oil. This representation of the experimental results clearly shows that the MSD measured in seeds reaches a plateau value at long diffusion time while in bulk oil the MSD was proportional to time as expected for free diffusion. Free diffusion is observed when molecules never meet physical barriers restricting their displacements during the diffusion time Δ. In this case, D_{meas} is an actual self-diffusion coefficient independent of the delay Δ and the mean squared displacement $\langle r^2 \rangle$ obeys $\langle r^2 \rangle = 6D_{meas}\Delta$ (Price 1997). This property is verified for the sunflower oil (Fig. 4), for which $\langle r^2 \rangle$ is roughly equal to 70 μm^2 for $\Delta = 1$ s, which is in fact a mean squared displacement small enough to be considered as a nonconfined displacement at the millimeter scale of the NMR tube.

Data reported in Fig. 4 reveal similar diffusion properties at very short diffusion times for sunflower oil, walnut, and sunflower seeds. We conclude that quasifree diffusion operates for TAG in these oil bodies within this time scale. This suggests that, for MSD smaller than OB sizes, the translational mobility of TAG molecules remains that of molecules in oil. This result was corroborated by the longitudinal relaxation properties; indeed, very similar T_1 values (0.5–0.6 s) were found for TAG molecules in seeds and in bulk oil. This means that fast molecular motions were not slowed down by this confinement level. Our conclusion is that the oil viscosity in oil bodies is unaffected by the confinement constraint. For long diffusion times, the mean squared displacements of TAG in seeds reach a plateau regime. The crossover between the free diffusion regime and the confined one occurs at a diffusion time even shorter than the limit value of the MSD. In the case of walnut seeds, we observe a more gradual trend to the plateau regime that is consistent with a larger polydispersity of OB size for this seed than for the other ones. To assess this hypothesis, the use of stronger gradient would be necessary (Guillermo and Bardet 2007).

In order to comment on the general behavior of the diffusion properties arising from these restricted diffusion conditions, a schematic description is presented below (Fig. 5a–c). The meaning of the PFGNMR experiment depends on the value of the dimensionless number $D\Delta/a^2$ that compares the molecular displacement expected in case of free diffusion with the typical size a of confinement domains (Price 1997; Guillermo and Bardet 2007).

1. For low $D\Delta/a^2$ values, the molecular motion is free of restriction and an actual self-diffusion coefficient is measured by the PFGNMR experiment $D_{meas} = D$ (Fig. 5a).
2. For intermediate values of $D\Delta/a^2$, the probability of contact with the confinement barrier increases with diffusion time Δ (Fig. 5b). This looks like a progressive slowing down of the molecule at the measurement time scale. The diffusion coefficient calculated with Eq. 1 is smaller than D and decreases with Δ, yielding a curvature of the plot of $D_{meas}\Delta$ versus Δ. This curvature was described by an exponential function of the number $D\Delta/a^2$ (Guillermo and Bardet 2007).
3. For large values of $D\Delta/a^2$, the conditions for confined diffusion are reached (Fig. 5c). The mean squared displacement $\langle r^2 \rangle$ cannot exceed the physical limits of the confinement volume. The product $D_{meas}\Delta$ tends toward a plateau, the value of which depends on the volume geometry. In case of confinement in spherical domains of radius a, the limit value of the product $D_{meas}\Delta$ is $a^2/5$ (Tanner and Stejskal 1968). Thus, at long diffusion times, Eq. 1 is rewritten as

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Table 1 OB diameters for seed of four species calculated from PFGNMR experiments in Fig. 4

Seeds	% TAG/FW	Δ range (s)	$D_{meas}\Delta$ (m^2)	OB diameter (μm)
Walnut	73	0.7–1	$(9.75 \pm 0.04) \times 10^{-13}$	4.42 ± 0.01
Sunflower	59	0.4–1	$(3.33 \pm 0.04) \times 10^{-13}$	2.58 ± 0.017
Sesame	56	0.25–0.7	$(2.57 \pm 0.05) \times 10^{-13}$	2.27 ± 0.02
Mustard	49	0.2–0.6	$(2.94 \pm 0.15) \times 10^{-14}$	0.77 ± 0.015

"Δ range" indicates the plateau domain where the average and standard deviation values of $D_{meas}\Delta$ were calculated. OB diameters are given by the relation $d_{nmr} = 2(5\ D_{meas}\Delta)^{1/2}$ arising from Eqs. 1 and 2

$$\underset{\Delta\to\infty}{I/I_0} = \exp\left(-(\gamma_H g\delta)^2\ \frac{a^2}{5}\right). \quad (2)$$

Applying this schematic description to TAG within seed oil bodies, the reference number $D\Delta/a^2$ can be estimated from the measured value of TAG diffusivity in oil, typically $D \approx 10^{-11}$ m^2 s^{-1}, and the oil body sizes published by Tzen et al. (1993). The OB radius a is given on the order of one micrometer, yielding $D\Delta/a^2 \approx 1$ for $\Delta \approx 0.1$ s for TAG in OBs. Thus, to observe the free–confined crossover of diffusion properties, PFGNMR experiments were performed with a diffusion time range of 10 ms to 1 s (Fig. 4). The sizes of OBs were calculated according to the relationship that exists at long diffusion times between the experimental plateau value of $D_{meas}\Delta$ and the radius a of the lipid domains of OBs: $a^2/5 = D_{meas}\Delta$ (Fig. 5c, Eq. 2). However, as shown by microscopy or light diffusion, the system of oil bodies is characterized by polydisperse values of OB radius, with a log-normal distribution often being observed (Tzen et al. 1993). The NMR signal of a given OB is proportional to the number of TAG molecules inside it. In other words, the intensity of the NMR signal due to an OB is proportional to its volume. Thus, the plateau value of the product $D_{meas}\Delta$ leads to the measurement of a volume-weighted mean value of the OB size distribution (Guillermo and Bardet 2007). Table 1 gives the OB diameters obtained from the results of PFGNMR experiments reported in Fig. 4. Thus, the mean diameters d_{nmr} range from 0.8 to 4.4 μm. As mentioned in the experimental part, these experiments were feasible because the ^1H longitudinal relaxation times of vegetable oils are long enough (0.5–0.6 s at 500 MHz) in comparison with the investigated range of diffusion times. In addition, the available gradients were strong enough to characterize micrometric confinement. Let us note that the PFGNMR measurement depends on the squared size, which increases the sensitivity of this method to determine the OB size.

For comparison with literature data, both PFGNMR and microscopy results are shown in Fig. 6. OB diameters d_{nmr} of sesame and mustard seeds are compared with values given by Tzen et al. (1993). The sunflower seed

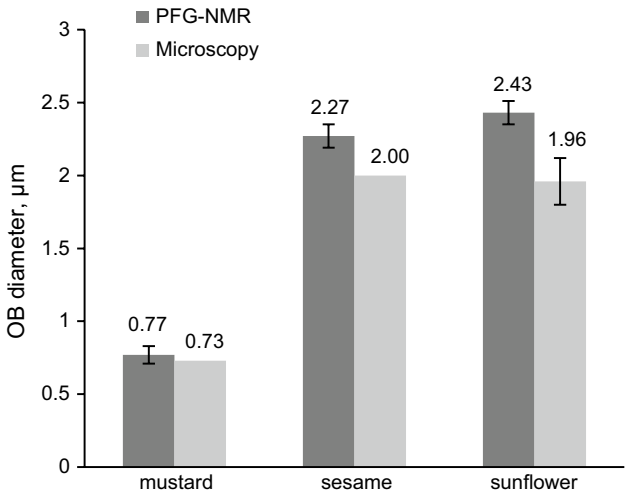

Fig. 6 Comparison of diameter values obtained by PFGNMR and data obtained by microscopy from the literature for mustard (*Brassica juncea* L.) and sesame (*Sesamum indicum* L.) (Tzen et al. 1993), and sunflower 'Dakar' cultivar (Mantese et al. 2006). For sunflower, the comparison was done for a given TAG amount (~56 % TAG/DW, i.e., 53 % TAG/FW). For NMR, error bars were calculated from three independent measurements. For microscopy, the error bar given by Mantese et al. was used

containing ~56 % TAG/DW is compared with that of sunflower 'Dakar' cultivar (Mantese et al. 2006) in order to compare seeds with alike amounts of TAG. As mentioned previously, the average size obtained by PFGNMR is weighted by the volume of the vesicles. This implies that the PFGNMR method leads to a mean value larger than the arithmetic mean of diameter values. Such is the case for sunflower seeds, whose average OB size obtained by PFGNMR is compared with the arithmetic mean determined from microscopy data (Mantese et al. 2006). However, when microscopy data are used to calculate the diameter of the average volume (Tzen et al. 1993), the agreement is improved as shown for the example of mustard and sesame seeds. The agreement between the two methods demonstrates that a valuable in vivo characterization of OB sizes can be obtained using weak field gradients.

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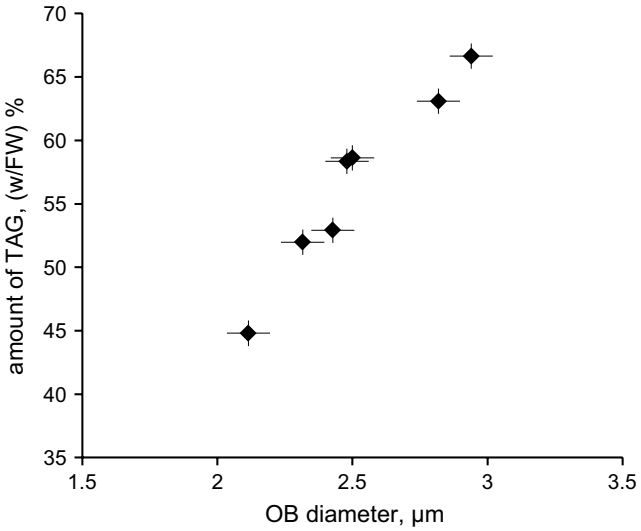

Fig. 7 Relationship between mean diameter of oil bodies and TAG content for a series of seven sunflower seeds. TAG content is in % (w/w) of fresh seed weight (FW)

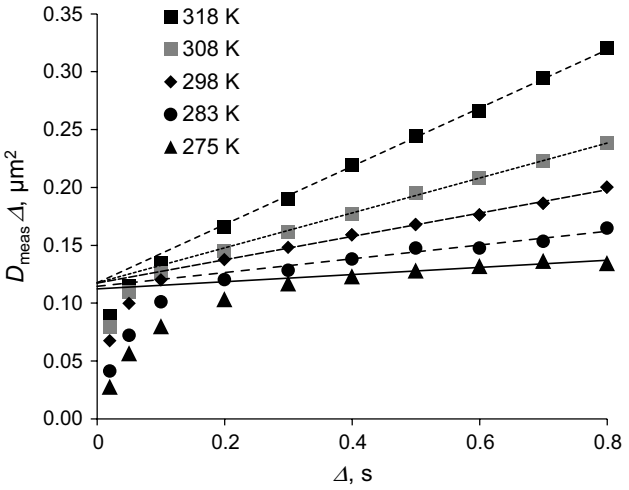

Fig. 8 Diffusion of TAG in lettuce seeds at different temperatures. The MSD $D_{meas}\Delta$ is plotted versus the diffusion time Δ

Correlation between oil body size and triacylglycerol amount

The PFGNMR measurements for the sunflower seeds containing 59 and 53 % TAG/FW give different OB diameters of 2.58 and 2.43 µm, respectively (Table 1; Fig. 6). As mentioned by several groups (Mantese et al. 2006; Jolivet et al. 2013), OB size can be correlated to the total amount of TAG. In order to illustrate this point, we studied a series of commercial sunflower seeds with variable amounts of TAG. The NMR methods were applied to determine both the lipid amount in seeds and the size of their oil bodies. Using experimental conditions presented in the "Materials and methods" section, NMR quantification of TAG was obtained by integration of their ^{1}H NMR spectra in order to determine the amount of TAG for each seed. In Fig. 7, mean OB diameters are plotted versus the weight to weight percentage of TAG in the fresh seeds. It is clear that the OB diameters increase with the amount of TAG. Very interestingly, this result is consistent with previous works based on microscopy and light scattering studies of isolated oil bodies extracted from sunflower (Mantese et al. 2006) and rapeseed (Jolivet et al. 2013).

The amount of TAG is proportional to the volume of OBs, i.e., proportional to the product $N_{OB}d^3_{nmr}$ with N_{OB} being the number of OBs. However, experimentally we observe a quasilinear dependence between the amount of lipids and the mean diameter. This means that, in these mature seeds, N_{OB} decreases as the square of the size when the total amount of TAG is increasing in the range 45–65 %. This therefore indicates that an increase in the amount of TAG in the mature seeds leads to a decreasing number of increasingly large OBs.

Long-range displacements of TAG through OB network

The plateau dependence previously shown (Fig. 4) should not be considered as a systematic behavior. In fact, we observed several examples for which the plateau regime was never reached. For some species, a linear increase of the mean squared displacement of TAG is observed instead of the flat dependence; for instance, the results obtained for lettuce seeds are shown in Fig. 8. In this example, the observed slopes appear to be temperature dependent, and they were shown to be perfectly reversible in this temperature range. This linear increase means that an additional free self-diffusion process is observed for long diffusion times Δ corresponding to long-range MSD. However, we observed that the extrapolations of these linear dependences at $\Delta = 0$ converge to the same displacement value that corresponds to the oil bodies size. This means that the random walk of TAG inside oil bodies and their additional mean squared displacement are two independent processes. Accordingly, the long-range TAG displacement does not prevent the determination of the oil bodies size (OB diameter is 1.54 ± 0.05 µm).

A random motion of the oil bodies themselves cannot be considered as a relevant explanation for this additional mobility. Indeed, the strong packing of OBs in mature seeds as observed by microscopy (Walters et al. 2005; Mantese et al. 2006; Chua et al. 2008; Hu et al. 2013) and the quasisolid feature of the matrix containing OB as shown by solid-state NMR spectroscopy (Bardet et al. 2001) are arguments against the OB mobility hypothesis. Moreover, the activation energy calculated from the temperature dependence of this long-range diffusion coefficient is equal to 33 kJ mol^{-1}, within the temperature range 275–318 K. This is close to the values found for

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sunflower and walnut crude oils of 38 and 30 kJ mol^{-1}, respectively. Consequently, this long-range diffusion process measured in mature seeds can be undoubtedly attributed to TAG themselves and not to any OB displacement. PFGNMR experiments reported in Fig. 8 show that this long-range diffusion coefficient (a few 10^{-13} m^2 s^{-1}) is much smaller than the corresponding coefficient in crude oil (about 10^{-11} m^2 s^{-1}). Therefore, to explain this additional free displacement of TAG, we propose the occurrence of inter-OB connectivity with a slow exchange of molecules between neighboring reservoirs (Hindmarsh et al. 2005; Sanders et al. 2009). This possibility is supported by the observation of OB fusion reported in literature (Leprince and Hoekstra 1998; Hsieh and Huang 2004; Miquel et al. 2014). Further investigations will be necessary to better understand the origin of this additional diffusion process.

Conclusions and perspectives

The present work demonstrates that relevant information about the average size of oil bodies inside seeds can be obtained directly without specific preparation of the sample by using PFGNMR with weak field gradients. The breakthrough of this method we propose for characterizing OBs in seeds is its suitability for use with standard liquid-state NMR spectrometers. In fact, the magnetic field gradient available is strong enough to record the first percents of the PFGNMR attenuation function, allowing the determination of a mean diameter of the micrometric oil bodies. It is worth noting that properties of OB membranes can also be inferred due to the in vivo feature of the PFGNMR method. This reveals evidence of TAG transfer through the network of interconnected OBs, which is dependent on the ability of adjacent membranes to open diffusion routes between OBs. Numerous Δ values are not necessary to evidence the existence of a plateau regime of TAG displacements in order to measure the OB mean size. For routine studies a few (4–5) long Δ values may be sufficient. The acquisition of PFGNMR attenuation functions at different diffusion delays Δ can be fully automated, and a sample changer can be used to analyze series of seeds. The PFGNMR method for OB size determination can therefore be a very rapid method, around 15–20 min per sample, that can be adapted to high-flow NMR studies. Taking into account the fact that the PFGNMR method does not require any preparation of the seed samples, it is clear that combining NMR spectroscopy and PFGNMR appears to be a powerful tool for rapid screening of TAG structures and OB size in oleaginous seeds.

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References

Atabani AE, Silitonga AS, Ong HC, Mahlia TMI, Masjuki HH, Badruddin IA, Fayaz H (2013) Non-edible vegetable oils: a critical evaluation of oil extraction, fatty acid compositions, biodiesel production, characteristics, engine performance and emissions production. Renew Sustain Energy Rev 18:211–245

Bardet M, Foray MF, Bourguignon J, Krajewski P (2001) Investigation of seeds with high-resolution solid-state C-13 NMR. Magn Reson Chem 39:733–738

Bardet M, Foray MF, Guillermo A (2006) High-resolution solid-state NMR as an analytical tool to study plant seeds. In: Webb GW (ed) Modern magnetic resonance part 3: applications in material science and food science, vol 3. Springer, Dordrecht, pp 1755–1759

Carlton KJ, Halse MR, Maphossa AM, Mallett MJD (2001) NMR stray-field analysis of oil drop size distribution in peanut cotyledons. Eur Biophys J 29:574–578

Chapman KD, Dyer JM, Mullen RT (2012) Biogenesis and functions of lipid droplets in plants. J Lipid Res 53:215–226

Chua ACN, Jiang PL, Shi LS, Chou WM, Tzen JTC (2008) Characterization of oil bodies in jelly fig achenes. Plant Physiol Biochem 46:525–532

Cotts RM, Hoch MJR, Sun T, Markert JT (1989) Pulsed field gradient stimulated echo methods for improved NMR diffusion measurements in heterogeneous systems. J Magn Reson 83:252–266

Debarre D, Supatto W, Pena AM, Fabre A, Tordjmann T, Combettes L, Schanne-Klein MC, Beaurepaire E (2006) Imaging lipid bodies in cells and tissues using third-harmonic generation microscopy. Nat Methods 3:47–53

Fleischer G, Skirda VD, Werner A (1990) NMR-investigation of restricted self-diffusion of oil in rape seeds. Eur Biophys J 19:25–30

Fordham EJ, Gibbs SJ, Hall LD (1994) Partially restricted diffusion in a permeable sandstone: observations by stimulated echo PFG NMR. Magn Reson Imaging 12:279–284

Fuchs J, Neuberger T, Rolletschek H, Schiebold S, Nguyen TH, Borisjuk N, Borner A, Melkus G, Jakob P, Borisjuk L (2013) A noninvasive platform for imaging and quantifying oil storage in submillimeter tobacco seed. Plant Physiol 161:583–593

Guillermo A, Bardet M (2007) In situ pulsed-field gradient NMR determination of the size of oil bodies in vegetable seeds. Analysis of the effect of the gradient pulse length. Anal Chem 79:6718–6726

Hindmarsh JP, Su JH, Flanagan J, Singh H (2005) PFG-NMR analysis of intercompartment exchange and inner droplet size distribution of W/O/W emulsions. Langmuir 21:9076–9084

Hsieh K, Huang AHC (2004) Endoplasmic reticulum, oleosins, and oils in seeds and tapetum cells. Plant Physiol 136:3427–3434

Hu ZY, Hua W, Zhang L, Deng LB, Wang XF, Liu GH, Hao WJ, Wang HZ (2013) Seed structure characteristics to form ultrahigh oil content in rapeseed. PLoS ONE 8:1–8

Huang AHC (1992) Oil bodies and oleosins in seeds. Annu Rev Plant Physiol Plant Mol Biol 43:177–200

Huang AHC (1996) Oleosins and oil bodies in seeds and other organs. Plant Physiol 110:1055–1061

Jolivet P, Deruyffelaere C, Boulard C, Quinsac A, Savoire R, Nesi N, Chardot T (2013) Deciphering the structural organization of the oil bodies in the Brassica napus seed as a mean to improve the oil extraction yield. Ind Crops Prod 44:549–557

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Leprince O, Hoekstra FA (1998) The responses of cytochrome redox state and energy metabolism to dehydration support a role for cytoplasmic viscosity in desiccation tolerance. Plant Physiol 118:1253–1264

Mantese AI, Medan D, Hall AJ (2006) Achene structure, development and lipid accumulation in sunflower cultivars differing in oil content at maturity. Ann Bot 97:999–1010

Miquel M, Trigui G, d'Andrea S, Kelemen Z, Baud S, Berger A, Deruyffelaere C, Trubuil A, Lepiniec L, Dubreucq B (2014) Specialization of oleosins in oil body dynamics during seed development in arabidopsis seeds. Plant Physiol 164:1866–1878

Morris KF, Johnson CS (1992) Diffusion-ordered 2-dimensional nuclear-magnetic-resonance spectroscopy. JACS 114:3139–3141

Murphy DJ (1993) Structure, function and biogenesis of storage lipid bodies and oleosins in plants. Prog Lipid Res 32:247–280

Murphy DJ, Hernandez-Pinzon I, Patel K (2001) Role of lipid bodies and lipid-body proteins in seeds and other tissues. J Plant Physiol 158:471–478

Neuberger T, Sreenivasulu N, Rokitta M, Rolletschek H, Gobel C, Rutten T, Radchuk V, Feussner I, Wobus U, Jakob P, Webb A, Borisjuk L (2008) Quantitative imaging of oil storage in developing crop seeds. Plant Biotechnol J 6:31–45

Odonnell DJ, Ackerman JJH, Maciel GE (1981) Comparative study of whole seed protein and starch content via cross polarization magic angle spinning C-13 nuclear magnetic-resonance spectroscopy. J Agric Food Chem 29:514–518

Pinzi S, Garcia IL, Lopez-Gimenez FJ, de Castro MDL, Dorado G, Dorado MP (2009) The ideal vegetable oil-based biodiesel composition: a review of social, economical and technical implications. Energy Fuels 23:2325–2341

Price WS (1997) Pulsed-field gradient nuclear magnetic resonance as a tool for studying translational diffusion. 1. Basic theory. Concepts Magn Reson 9:299–336

Price WS (1998) Pulsed-field gradient nuclear magnetic resonance as a tool for studying translational diffusion: Part II: Experimental aspects. Concepts Magn Reson 10:197–237

Russo D, Dassisti M, Lawlor V, Olabi AG (2012) State of the art of biofuels from pure plant oil. Renew Sustain Energy Rev 16:4056–4070

Sacchi R, Addeo F, Paolillo L (1997) ^{1}H and ^{13}C NMR of virgin olive oil. An overview. Magn Reson Chem 35:S133–S145

Samii-Saket G, Boersma JG, Ablett GR, Falk DE, Fletcher R, Rajcan I (2011) Single soybean seed NMR calibration for oil measurement using commercial cooking oils. J Am Oil Chem Soc 88:1795–1798

Sanders M, Mueller R, Menjoge A, Vasenkov S (2009) Pulsed field gradient nuclear magnetic resonance study of time-dependent diffusion behaviour and exchange of lipids in planar-supported lipid bilayers. J Phys Chem B 113:14355–14364

Stejskal EO, Tanner JE (1965) Spin diffusion measurements: spin echoes in presence of a time-dependent field gradient. J Chem Phys 42:288–292

Tanner JE, Stejskal EO (1968) Restricted self-diffusion of protons in colloidal systems by pulsed-gradient spin-echo method. J Chem Phys 49:1768–1777

Terskikh VV, Feurtado JA, Borchardt S, Giblin M, Abrams SR, Kermode AR (2005) In vivo C-13 NMR metabolite profiling: potential for understanding and assessing conifer seed quality. J Exp Bot 56:2253–2265

Tzen JTC, Cao YZ, Laurent P, Ratnayake C, Huang AHC (1993) Lipids, proteins, and structure of seed oil bodies from diverse species. Plant Physiol 101:267–276

Walters C, Landre P, Hill L, Corbineau F, Bailly C (2005) Organization of lipid reserves in cotyledons of primed and aged sunflower seeds. Planta 222:397–407

Wu DH, Chen AD, Johnson CS (1995) An improved diffusion-ordered spectroscopy experiment incorporating bipolar-gradient pulses. J Magn Reson Ser A 115:260–264

Zakhartchenko NL, Skirda VD, Valiullin RR (1998) Self-diffusion of water and oil in peanuts investigated by PFG NMR. Magn Reson Imaging 16:583–586